Cloning and Characterization of the *Tribolium castaneum* Eye-Color Genes Encoding Tryptophan Oxygenase and Kynurenine 3-Monooxygenase

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ABSTRACT

The use of eye-color mutants and their corresponding genes as scorable marker systems has facilitated the development of transformation technology in Drosophila and other insects. In the red flour beetle, *Tribolium castaneum*, the only currently available system for germline transformation employs the exogenous marker gene, *EGFP*, driven by an eye-specific promoter. To exploit the advantages offered by eye-pigmentation markers, we decided to develop a transformant selection system for Tribolium on the basis of mutant rescue. The Tribolium orthologs of the Drosophila eye-color genes *vermilion* (*tryptophan oxygenase*) and *cinnabar* (*kynurenine 3-monooxygenase*) were cloned and characterized. Conceptual translations of *Tc vermilion* (*Tcv*) and *Tc cinnabar* (*Tccn*) are 71 and 51% identical to their respective Drosophila orthologs. We used RNA interference (RNAi) to show that *T. castaneum* larvae lacking functional *Tcv* or *Tccn* gene products also lack the pigmented eyespots observed in wild-type larvae. Five available eye-color mutations were tested for linkage to *Tcv* or *Tccn* via recombinational mapping. No linkage was found between candidate mutations and *Tccn*. However, tight linkage was found between *Tcv* and the white-eye mutation *white*, here renamed *vermilion*[white] (*v*[w]). Molecular analysis indicates that 80% of the *Tcv* coding region is deleted in *v*[w] beetles. These observations suggest that the Tribolium eye is pigmented only by ommochromes, not pteridines, and indicate that *Tcv* is potentially useful as a germline transformation marker.

RECENT advances in genomics and bioinformatics promise to hasten the discovery and analysis of genes that regulate important biological phenomena. Such efforts will be aided by continued development of transposon-based systems for the experimental manipulation of target genomes. The red flour beetle, *Tribolium castaneum*, is an excellent candidate in which to develop such technology because of its long history as an experimental subject for genetic analyses, its ease of genetic manipulation, its prominence as a pest species, and its potential as a model for other Coleopteran pest species. To further improve the utility of the Tribolium genetic system, transposon-based procedures analogous to those based on the *P*-transposable element in Drosophila are needed. The *P* element has been harnessed and tailored for germline transformation applications such as mutagenesis by transposon tagging and enhancer trapping. However, useful *P*-element function is restricted to the genus Drosophila (O'Brochta and Handler 1988). A summary of recent nondrosophilid insect transformation efforts can be found in Atkinson *et al.* (2001).

Recently Berghammer *et al.* (1999) achieved germline transformation in *T. castaneum*. The authors utilized both the *Hermes* and *piggyBac* transposable elements marked with a gene encoding an enhanced green fluo-rescent protein (EGFP) driven by the eye-specific promoter 3xP3. This system (Horn *et al.* 2000) shows great promise, but is limited by the cell autonomy of EGFP expression, the technical demands of EGFP detection, and the tissue specificity of the 3xP3 promoter. To overcome these potential limitations, we are exploring the use of eye-color genes as transformation markers. Eye-color markers have been used successfully in Drosophila since the advent of *P*-mediated germline transformation (Rubin and Spradling 1982). These genes generate an easily scored visible phenotype when introduced into the appropriate mutant background. The use of such genes eliminates the need for specialized detection systems, thus making transformation-based protocols more widely accessible. In addition, the availability of several different transformant selection systems allows greater flexibility in the design of sophisticated protocols.

A large number of mutations are known to affect the pigmentation of the compound eye of Drosophila, including those that affect the biosynthesis or transport of ommochrome (brown) and pteridine (red) pigments. While some insects lack pteridine pigments (*e.g., Anopheles gambiae*, Beard *et al.* 1995), ommochrome pigments have been found in all insects examined to date. Early work with Drosophila eye-color mutants revealed that *vermilion* (*v*) and *cinnabar* (*cn*) are involved in ommochrome production (Beadle and Ephrussi 1937). The *vermilion* gene encodes tryptophan oxygenase (TO; Baglioni 1959; Ballie and Chovnick 1971; Walker *et al.*

1986), an enzyme that converts tryptophan to formylky-nurenine (see reviews by LINZEN 1974; PHILLIPS and FOR-REST 1980), whereas *cinnabar* encodes kynurenine 3-mono-oxygenase (KM; GHOSH and FORREST 1967; WARREN *et al.* 1996), which converts kynurenine to 3-hydroxy-kynurenine (GHOSH and FORREST 1967). Mutations in *v* or *cn* rarely cause a decrease in fitness, and overexpression of these genes is not deleterious.

Such eye-color genes offer another important advantage. Unlike EGFP, the *v* and *cn* genes are not limited by cell autonomy. The importance of this is realized when transgene insertion occurs in or near a heterochromatic region. Transposition into such chromosomal sites often leads to position-effect variegation (random gene silencing), evidenced by a characteristic mosaic pattern of expression in the case of cell-autonomous genes. However, since TO and KM and their pigment products are transported across cell membranes their effects can be manifest even in the absence of expression in the target tissue. For example, *rosy* mutant flies, mosaic for cells bearing a *rosy*-marked *P* element, have wild-type eye color even in the absence of *rosy* gene expression in the cells of the eye (REAUME *et al.* 1989). Although these flies were true somatic mosaics rather than germline transformants showing variegation, the result (global effect from local expression) is the same. In contrast, in the cell-autonomous 3xP3-EGFP system, transgene expression in the eye is required for transformant selection.

A transformant selection system of this type requires a cloned functional eye-color gene and a corresponding loss-of-function mutant strain. Here we report the cloning and characterization of the Tribolium orthologs of *vermilion* (*Tcv*) and *cinnabar* (*Tccn*). We also report the identification of a white-eyed strain carrying a *Tcv* null mutation.

MATERIALS AND METHODS

Strains: The wild-type strains used in this work were as follows: (1) GA-1, a North American strain described by HALISCAK and BEEMAN (1983); (2) GA-2, a near-homozygous inbred derivative of GA-1 (S. THOMPSON, J. STUART and S. BROWN, unpublished data); and (3) T-1, an Indian strain described by THOMPSON *et al.* (1995). Wild-type flour beetles have pitch-black eye color. The recessive white-eyed mutation, *white* (*w*, EDDLEMAN and BELL 1963), is shown in this work to be an allele of *Tcv* and is hereafter referred to as *vermilion^white^* (*v^w^*). Other eye-color mutant strains used in this work are shown in Table 1. Beetles were reared in yeast-fortified wheat flour under standard conditions (BEEMAN *et al.* 1986).

Degenerate PCR: Nested, degenerate primers were designed on the basis of highly conserved regions of the TO/Vermilion proteins (underlined in Figure 3). First-round PCR was performed using 6 ng of GA-1 genomic DNA with CAY GAYGARCAY and CCARAARTTRAANCC (all primer sequences are shown 5′–3′). Using 1 μl of first-round product as template, a second round of PCR was performed with the nested primers TAYGARYTNTGGTTYAARCA and CCNGG NGTNCKYTCNARCC to generate the pGv1 fragment.

A similar strategy was used to amplify a portion of the Tribolium *cinnabar* ortholog. Nested degenerate primers were

designed on the basis of highly conserved regions of the KM/Cinnabar proteins (underlined in Figure 6). First-round primers were AAYTAYYTNCAYATHTGGCC and RTARTTRTACA TNGC, while the nested primers ACNTTYATGATGATHGC and CCNGCRTTCATNCCYTGNCC were used for a second round of PCR to generate the pGcn1 fragment.

Isolation of cDNA and genomic clones: Approximately 3.6 ng of purified DNA from a *T. castaneum* embryonic cDNA library (see SHIPPY *et al.* 2000) was used as template for rapid amplification of cDNA ends (RACE) using *Tcv* or *Tccn* gene-specific primers paired with pCMVSPORT4 vector primers. Nested, gene-specific primers were designed from the sequences of the pGv1 and pGcn1 clones. To obtain the 5′ region of each cDNA, nested, gene-specific primers were used in conjunction with the SP6 vector primer. Similarly, the 3′ ends of the *Tcv* and *Tccn* cDNAs were amplified using nested, gene-specific primers in conjunction with the T7 vector primer. Inserts of resulting clones were sequenced. Primers from the 5′ and 3′ untranslated regions (UTRs) were used to amplify *Tcv* (pCv) and *Tccn* (pCcn) cDNAs that included complete coding regions.

A GA-2 *T. castaneum* bacterial artificial chromosome (BAC) library constructed in pBACe3.6 (a gift from the Exelixis Pharmaceutical Co., South San Francisco) was screened to obtain full-length genomic clones of *Tcv* and *Tccn*. The *Tcv* and *Tccn* degenerate PCR products, pGv1 and pGcn1, were individually radiolabeled (Prime-It; Stratagene, La Jolla, CA). See Figures 1A and 4A for the size and location of probes. After prehybridization for 3 hr at 65° in 5× Denhardt's buffer (0.5% SDS, 5× SSC, and 20 μg/μl herring sperm DNA) the filter was hybridized overnight at 65° with a mixture of the two ³²P-labeled probes in fresh hybridization buffer and washed in 2× SSC, 0.1% SDS at 65°. Full-length *Tcv* and *Tccn* clones were subsequently identified by PCR using gene-specific primers.

Northern analysis: Northern analysis of *Tcv* was performed as previously described by SHIPPY *et al.* (2000), but using pupal rather than embryonic mRNA. The *Tcv* cDNA (pCv) was used as probe.

DNA sequencing and analysis: BAC DNA templates were sequenced using the ThermoSequenase kit (Amersham Life Sciences, Cleveland). PCR products were gel purified (Prep-A-Gene DNA purification systems; Bio-Rad Laboratories, Hercules, CA) and cloned using the pCRII-TOPO kit (Invitrogen, Carlsbad, CA). Sequences from plasmid clones were obtained using an ABI 373A DNA sequencer (DNA Sequencing Core Facility, College of Veterinary Medicine, Kansas State University) or an ABI 3700 DNA sequencer (Sequencing and Geno-typing Facility, Plant Pathology, Kansas State University). Data were analyzed using the MacVector sequence analysis program (Eastman Kodak Company, New Haven, CT).

RNA interference: The MEGAscript SP6 and T7 *in vitro* transcription kits (Ambion, Austin, TX) were used to generate sense and anti-sense RNA from the *Tcv* cDNA, pCv. The combined RNAs were heated to 100° and allowed to slowly cool to room temperature (BROWN *et al.* 1999). The same procedure was performed using the 1.66-kb *Tccn* genomic clone, pGcn. Double-stranded RNA from *Tcv* (1 μg/μl) or *Tccn* (0.5 μg/μl) was injected into wild-type (GA-1), 0- to 4-hr embryos (injection volume ~100 pl/embryo) in injection buffer (5 mM KCl, 1 mM KPO₄, and 1% v/v green food coloring, pH 6.8). Injected embryos were incubated at room temperature for 1 week in a humidified, oxygenated chamber (Billups-Rothenberg, Del Mar, CA). Late-stage embryos and first instar larvae were screened for larval eyespot pigmentation.

Recombinational mapping: Single-pair crosses were set up between GA-1 or T-1 virgin females and *chestnut, hazel, platinum, ruby,* or *v^w^* males. A single F₁ virgin female from each line was backcrossed to the homozygous recessive male parent.

List of Tribolium eye-color mutants

Strains[a]	Adult eye color	Larval eyespot	Linkage group	Complementation group	References
Wild type	Black	++++	NA	NA	NA
bronze (*bz*)	Near WT	+++	Not X	*bz*	R. Beeman (unpublished data)
chocolate	Near WT	+++	Not X	*bz*	R. Beeman (unpublished data)
chestnut (*c*)	Red	+	7	*c*	Eddleman and Bell (1963)
hazel (*h*)	Red	+	4	*h*	Dawson (1967)
ruby (*rb*)	Red	+	5	*rb*	Dewees (1963)
red^{Z2}	Red	+	9	*i*	R. Beeman (unpublished data)
red-1 (*r*)	Red	+	X	*r*	Eddleman and Bell (1963)
peach	Peach-red	−	X	*r*	R. Beeman (unpublished data)
pink Ndg	Light red/pink	−	X	*r*	R. Beeman (unpublished data)
pink Tiw	Light red/pink	−	X	*r*	R. Beeman (unpublished data)
ivory (*i*)	Cream	−	9	*i*	Bartlett and Bell (1966)
platinum (*pte*)	White	−	X	*pte*	Yamada (1961)
pearl (*p*)	White	−	9	*p*	Park (1937)
vermilion^{white} (*v^w*)	White	−	4	*v^w*	Eddleman and Bell (1963)

[a] Order of strains is based on the intensity of adult eye pigmentation. Strains were also scored for relative degree of larval eyespot pigmentation and include the following: ++++, eyespots black; +++, eyespots nearly equivalent to that of wild type; +, eyespots with reduced pigmentation; and −, eyespots that lack discernible pigment.

The progeny from each backcross were sorted by eye-color phenotype as late-stage pupae, and single-beetle DNA isolations were performed using the Wizard Genomic DNA isolation kit (Promega, Madison, WI) according to the manufacturer's protocol. PCR products obtained using the *Tcv*-specific, exon 1 primer GACAGGTCGTAATGAGTTGCCCAC and the exon 3 primer ACGTCGCTGAAAATGTTG were analyzed on 2% agarose gels (for the *v^w* backcross) or Novex precast 4–20% polyacrylamide TBE gels (for all others; Invitrogen). PCR products obtained using *Tccn* BAC-specific primers (ACGGG GTGGTCCATGAGTAATAA and TGAGGCGGCACAGAGAT) were analyzed on Novex precast 4–20% polyacrylamide TBE gels (Invitrogen). Dimorphic markers were scored, and recombination frequencies were calculated.

Southern analysis: Two micrograms of genomic DNA was digested with *Eco*RI and electrophoresed overnight by field inversion gel electrophoresis (FIGE) on a 0.7% agarose gel. The DNA was transferred to a GeneScreen membrane (New England Nuclear Life Sciences, Boston), using a Turboblotter rapid downward transfer system (Schleicher & Schuell, Keene, NH). A ^{32}P-labeled, 1.6-kb *Tcv* fragment (pGv2) was hybridized to the membrane overnight in PerfectHybPlus (Sigma, St. Louis) at 68°. The membrane was stripped at 65° and hybridized with a ^{32}P-labeled, ~900-bp fragment containing the *Tcv* promoter and 5′ coding region. The membrane was stripped again and another hybridization was performed with a ^{32}P-labeled fragment containing the 3′ end of the *Tcv* coding region. All hybridizations were performed as described above.

Deletion breakpoint analysis: Eight nanograms of *v^w* genomic DNA served as template for the first round of universal PCR, using a mixture of five universal primers (Uni-4, Uni-5, Uni-7, Uni-8, and Uni-9) and GSP1, the *Tcv* exon 6 primer TAGACAAGGGGGGGATGTAG. The primary PCR reaction (1 μl) was used as template for a second round of PCR with M13 (−40) (the linker sequence at the 5′ ends of all five universal primers) and GSP2, the nested exon 6 primer CCGTGGTATCAAAAACGTC. The resulting PCR product was cloned and sequenced. For a complete list of the universal primer sequences and PCR conditions see Beeman and Stauth (1997).

RESULTS

Homology-based cloning of *Tcv*: Analysis of *Tcv* genomic and cDNA clones is shown in Figures 1 and 2. Degenerate primers based on conserved regions of *vermilion* orthologs amplified a 457-bp fragment from Tribolium genomic DNA (pGv1). On the basis of this genomic sequence, primers were designed for 5′ and 3′ RACE. The 5′ clone (pC5′v) yielded 220 bp of additional upstream sequence, encompassing an apparent translational start site and 20 bp of 5′ UTR. The 3′ cDNA fragment (pC3′v) provided 774 bp of downstream sequence and a poly(A) tail. Primers designed in the 5′ and 3′ UTRs were used to amplify a cDNA containing the entire coding sequence. This cDNA (pCv) is 1343
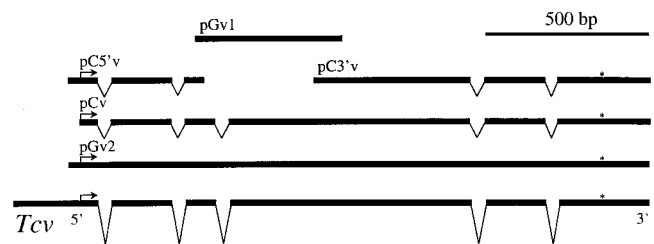

Figure 1.—*Tc vermilion* gene structure. This schematic indicates the location of the original *Tc vermilion* (*Tcv*) genomic clone (pGv1), as well as the *Tcv* cDNA clones (pC5′v, pC3′v, and pCv) and *Tcv* genomic clone (pGv2) in relationship to the *Tcv* locus. Arrows indicate the positions of the translational start sites, while asterisks mark the locations of the stop codons. Exons are represented as thick lines on cDNA clones. GenBank accession nos. are as follows: AYO52625 (pGv1), AYO52395 (pC5′v), AYO52394 (pC3′v), AYO52390 (pCv), and AYO52392 (pGv2).

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          -200                  -180                  -160                  -140
GCATCAGAATTTTTAAAATAAGTCCAGTTATTTGATAGCCGATAGTGTATTAGCTTAAGCAAATTACGTCA
          -120                  -100                  -80
TTTGCATCAGAATTTTTAAAATAAGTCCAGTTATTTGATAGCCGATAGTGTATTAGCTTAAGCAAATTACG
      -60                  -40                  -20                  +1
TCAGCTTGATTAAGTCAACGATCCAATCGAGTCAATATCGCTTTAGTTAGTCTAAATTTCAAGTTAAAAAC
          +20
GACAGGTCGTAATGAGTTGCCCACTGAGACCCTCgtaagtatgatttaatttaattattccatttacaatt

aatcgtaaattaagTGAAGCCCAAGAAGGCGACCAACTGAGCGAAGAATGTGGGATGCTTTACGGTGAATA

CTTGATGTTGGACAAGATTTTAGAAGCCCAAAGATTACTCAGCGAGCAAAATAACCAACCCGTGCACGACG

AGCATTTATTTATTGTGACCCATCAAGgtaaaaaacccacttaaccgtaattaactataacaagtaattcc

agCGTATGAGTTATGGTTCAAACAAATAATCTACGAGCTGGACTCGATCCGCAACATTTTCAGCGACGTTT

TAGAAGAGTCGCAAACTTTGGAAATCCTCAAACGCCTCAATCGTGTTGTTTTGATCCTCAAGgttcacaat

ttcgcaacaaattgcggtcacgagataaaactgtcgcaattgtagGTCTTGGTGGACCAAGTGATGATTCT

GGAAACGATGACACCGCTCGATTTCATGGACTTCCGGTGTTATCTGCGGCCCGCTTCCGGGTTCCAGAGCC

TGCAGTTCAGGCTCCTGGAAAACAAGCTCGGGGTGCGCCAGGAAAACCGCGTCAAATACAACCAGAACTAC

TCGAAAGTGTTCGGAAATGACGAAAAAGCGCTCGAGCAAATCGCCAAGTCGGAGAAAGAGCCGTCTTTGAC

CGATTTGGTCCAGCGGTGGCTGGAGCGAACGCCGGGGCTGGAGCTCGAGGGGTTTAATTTCTGGGGGAAGT

ACCAAAAAGCGGTCGAGAAGCTTTTAACCGAGCAAAAAGAGCTGGCGGAGAAGGAGGAGGCGGAGACTTTA

AAGCGTTACAAATTGAACGATTTGGAGAAGAGACGCGAGGTTTACGAGTCGATTTTTAAAGTTGAGGTCCA

TGAGGCTCTAATGTCGCGCGGCGAGAGGCGCTTCTCACACAAAGCCCTGCAAGGGGCTATCATGATCACCT

TTTACAGGGACGAACCCAGGTTCAGTCAGCCCCACCAAATTCTAACACTTTTGATGGACATAGACTCGCTG

ATTACGAAATGGCGGTgttagtaaatccgtacgtcaattgtttttgtttaaattttataatttcagATAAT

CATGTTTTGATGGTGCAAAGAATGATTGGTTCTTCGCAGTTAGGAACAGGGGGCTCTTCGGGGTATCAGTA

TTTAAGGTCAACGTTAAGgtgaaattgatttcaacacggttttttgcaatgtttgattttgttttttcagCGA

TCGGTATAAAGTTTTCGTGGACCTTTTCAATTTATCGACGTTTTTGATACCACGGTCCTACATCCCCCCCT

TGTCTACGTCTATGAGAAGTCATTTGTGCAATTGGGGGTCGGCGAATAGCACCAATAACGTGTCGAATGGA

AACAATTAATTATTTCGTTAAGTGAACGCAATTTTACTAATTGGACGCACTTATTTATTAATTTTTACTCA

CCTGAATTTGAATTTGCGGCACCTGTCACTTGTAAACTGTCAACTGTCAAATAATGTACAGTCTAGGACAC

ACTAAATGCACGGGGCAATTGAATAAAATTAA
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FIGURE 2.—Nucleotide sequence of the wild-type *Tc vermilion* locus. +1 indicates the start of the longest *Tcv* transcript detected. Proposed initiator sequences are underlined in the putative *Tcv* promoter (−200 to +20). Boldface type denotes a consensus downstream promoter element. Start and stop codons are boxed. The polyadenylation signal is double underlined and introns are shown in lowercase italics. All nucleotides upstream of the double-underlined base (in boldface type), following the last intron, are deleted in the *vermilion^white* allele. GenBank accession nos. are AYO52392 (pGv2) and AF419847 (putative *Tcv* promoter).

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Tc  MS-CPLRPSEAQEGDQLSEEC----------------GMLYGEYLMLDKILEAQRLLSEQ
Dm  MS-CPYAGNGNDHDDSAVPLTTEV-------------GKIYGEYLMLDKLLDAQCMLSEE
Ag  MS-CPMRSGFVDSVQGGHHLGSEA-------------GMLYGEYLMLDKVLSAQRMLSVE
Hs  MSGCPFLGNNFGYTFKKLPVEGSEEDKSQTGVNRASKGGLIYGNYLHLEKVLNAQELQSET


Tc  NNQPVHDEHLFIVTHQAYELWFKQIIYELDSIRNIFS--DVLEESQTLEILKRLNRVVLIL
Dm  DKRPVHDEHLFIITHQAYELWFKQIIFEFDSIRDMLDA-EVIDETKTLEIVKRLNRVVLIL
Ag  GKKPVHDEHLFIVTHQAYELWFKQIIFELDSIRDLFST-EHIEESRTLEILKRLNRIVMIL
Hs  KGNKIHDEHLFIITHQAYELWFKQILWELDSVREIFQNGHVRDERNMLKVVSRMHRVSVIL


Tc  KVLVDQVMILETMTPLDFMDFRCYLRPASGFQSLQFRLLENKLGVRQENRVKYN-QNYSKV
Dm  KLLVDQVPILETMTPLDFMDFRKYLAPASGFQSLQFRLIENKLGVLTEQRVRYN-QKYSDV
Ag  KLLVDQVPILETMTPLDFMDFRDYLSPASGFQSLQFRLLENKLGVKSEHRVKYN-QKYTEV
Hs  KLLVQQFSILETMTALDFNDFREYLSPASGFQSLQFRLLENKIGVLQNMRVPYNRRHYRDN


Tc  FGNDEKALEQIAKSEKEPSLTDLVQRWLERTPGLELEGFNFWGKYQKAVEKLLTEQKELAE
Dm  FS-DEEARNSIRNSEKDPSLLELVQRWLERTPGLEESGFNFWAKFQESVDRFLEAQVQSAM
Ag  FASDPGAIERIGTTETEPSLADLVQKWLERTPGLEQDGFNFWGKFQESVEQLLAEQEASAM
Hs  FK--GEENELLLKSEQEKTLLELVEAWLERTPGLEPHGFNFWGKLEKNITRGLEEEFIRIQ


Tc  KEEAETLKRYKLNDLEKRREVYESIFKVEVHEALMSRGERRFSHKALQGAIMITFYRDEPR
Dm  EEPVEKAKNYRLMDIEKRREVYRSIFDPAVHDALVRRGDRRFSHRALQGAIMITFYRDEPR
Ag  SEEHENVREYRLMDIDKRREVYKSIFDAQVHDALVARGERRFTHKALQGAIMITFYRDEPR
Hs  AKEESEEKEEQVAEFQKQKEVLLSLFDEKRHEHLLSKGERRLSYRALQGALMIYFYREEPR


Tc  FSQPHQILTLLMDIDSLITKWRYNHVLMVQRMIGSSQLGTGGSSGYQYLRSTLSDRYKVFV
Dm  FSQPHQLLTLLMDIDSLITKWRYNHVIMVQRMIGSQQLGTGGSSGYQYLRSTLSDRYKVFL
Ag  FSQPHQLLMLLMDIDSLITKWRYNHVIMVQRMIGSQQLGTGGSSGYQYLRSTLSDRYKVFL
Hs  FQVPFQLLTSLMDIDSLMTKWRYNHVCMVHRMLGSKA-GTGGSSGYHYLRSTVSDRYKVFV


Tc  DLFNLSTFLIPRSYIPPLSTSMRSHLCNWGSANSTNNVSNGNN---------------388
Dm  DLFNLSTFLIPREAIPPLDETIRKKLINKSV-------------------------379
Ag  DLFNLSTFLIPRQSIPPLTNEMQKALNLAWGSPAHFARNGSLH---------------392
Hs  DLFNLSTYLIPRHWIPKMNPTIHKFLYTAEYCDSSYFSSDESD---------------406
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FIGURE 3.—Multiple alignment of tryptophan oxygenase sequences from *Tribolium castaneum* (*Tc*; GenBank accession no. AYO52390), *Drosophila melanogaster* (*Dm*; GenBank accession no. A34780), *Anopheles gambiae* (*Ag*; GenBank accession no. AAC27659), and *Homo sapiens* (*Hs*; GenBank accession no. P48775). Residues that are identical in all four species are in boldface type. Regions used for designing degenerate primers are underlined.

bp in length and includes 11 bp of 5′ UTR, 1164 bp of open reading frame (ORF), and 168 bp of 3′ UTR. Alignment (PEARSON and LIPMAN 1988) of mammalian and insect TO proteins (Figure 3) shows that the conceptual translation of pCv is 71% identical to Drosophila Vermilion and 56% identical to human TO. Conserved motifs previously identified by alignment of TO/Vermilion orthologs are also present in the Tribolium protein.

Tcv gene structure: Northern analysis showed that a *Tcv* fragment (pCv) hybridized to a 1.55-kb transcript (pupal mRNA, data not shown), which corresponds to the length of the pCv cDNA (1343 bp), assuming a 200-bp poly(A) tail. Primers from the 5′ and 3′ UTRs of *Tcv* were used to amplify a genomic fragment (pGv2). Gene structure was determined by comparison of cDNA and genomic sequences (Figures 1 and 2). Both the number and location of introns are highly conserved between the Drosophila (SEARLES *et al.* 1990) and Tribolium *vermilion* genes. Both genes have five short introns that occur at identical or nearly identical positions. Both genes also appear to have very short 5′ UTRs. The longest *Tcv* 5′ UTR we could detect in Tribolium is 20 bp, while that of Drosophila is only 57 bp (FRIDELL and SEARLES 1992).

Since the 5′-most cDNA fragment contained only 20 bp of 5′ UTR, we sought to more accurately define the 5′ end of *Tcv* by identifying the promoter. A *T. castaneum* genomic BAC library was probed with pGv1, and one positive clone was selected for analysis. A sequencing primer from the 5′ UTR of *Tcv* was used to obtain 700 bp of upstream genomic sequence. This sequence was analyzed for the presence of promoter elements (Figure 2). No consensus TATA box was found, but several arthropod initiator sequences (Inrs; CHERBAS and CHERBAS 1993) were identified. Ten Inrs are located within −140 to +1 (+1 corresponds to the 5′-most nucleotide of pC5′v), and a consensus downstream promoter element (DPE) is located at +14 (SMALE 1997; Figure 2). FRIDELL and SEARLES (1992) used a series of deletion constructs to demonstrate that Drosophila *v* possesses important sequence motifs in the 5′ UTR (between +19 and +36 and between +47 and +57 downstream of the transcription start site), without which transcription of the fly gene is silenced. Comparison of the 5′ UTR of *v* to that of *Tcv* reveals similar motifs (data not shown). The presence in the *Tcv* 5′ UTR of a putative DPE of (A/G)G(A/T)CGT (SMALE 1997) and the lack of a TATA box within 700 bp upstream of the putative transcription start site suggest similarities between the promoters of fly and beetle *vermilion* genes (Figure 2). Taken together, the data suggest that pC5′v is derived from a full-length or nearly full-length transcript, and
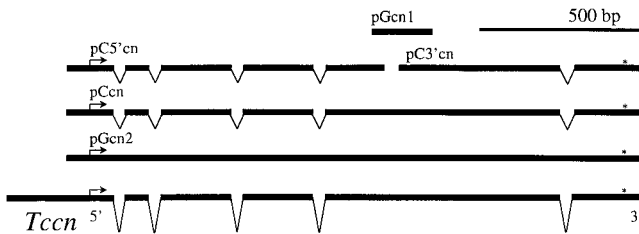
FIGURE 4.—*Tc cinnabar* gene structure. This schematic indicates the location of the original *Tc cinnabar* (*Tccn*) genomic clone (pGcn1), as well as the *Tccn* cDNA clones (pC5′cn, pC3′cn, and pCcn) and *Tccn* genomic clone (pGcn2) in relationship to the *Tccn* locus. Arrows indicate the positions of the translational start sites, while asterisks mark the locations of the stop codons. Exons are represented as thick lines on cDNA clones. GenBank accession nos. are as follows: AYO52623 (pGcn1), AYO52622 (pC5′cn), AYO52624 (pC3′cn), AYO52391 (pCcn), and AYO52393 (pGcn2).

that, like Drosophila *v* (SEARLES *et al.* 1990), *Tcv* is transcribed from a TATA-less promoter.

Homology-based cloning of *Tccn*: Analysis of *Tccn* genomic and cDNA clones is shown in Figures 4 and 5. PCR with degenerate primers based on conserved regions of *cinnabar* orthologs produced a 302-bp genomic fragment (pGcn1). Primers for 5′ and 3′ RACE were designed on the basis of this genomic sequence. The 5′ clone (pC5′cn) provided an additional 721 bp of upstream sequence, including an apparent translational start site and 50 bp of 5′ UTR. The 3′ cDNA fragment (pC3′cn) provided 468 bp of downstream sequence and a poly(A) tail. Primers from the 5′ and 3′ UTRs were used to amplify a cDNA containing the entire coding sequence. This cDNA (pCcn) is 1421 bp in length and includes 50 bp of 5′ UTR, a 1335-bp ORF, and 36 bp of 3′ UTR. Alignment (PEARSON and LIPMAN 1988) of mammalian and insect KM proteins (Figure 6) shows that the conceptual translation of pCcn is 53% identical to Bombyx KM, 51% identical to Drosophila KM, and 49% identical to human KM. All conserved motifs previously identified by alignment of KM/Cinnabar orthologs are present in the Tribolium protein.

***Tccn* gene structure:** A *Tccn* genomic fragment (pGcn2) was amplified and cloned using a method similar to that described above for *Tcv*. Gene structure was determined by comparison of cDNA and genomic sequences (Figures 4 and 5). Unlike *v*, the number and locations of introns are not well conserved between the Drosophila and Tribolium *cn* genes. *Tccn* contains five small introns (Figure 4) while Drosophila *cn* has only two (WARREN *et al.* 1996), with the location of only the first intron being conserved.

The longest ORF includes a methionine 11 residues downstream from a stop codon. However, it was possible that this clone did not represent the complete *Tccn* coding region, since the protein it encodes lacks ∼81 amino acids present in the Drosophila protein. To determine whether the *Tccn* ORF contained a complete cod-

ing region a *T. castaneum* genomic BAC library was probed with pGcn1, and a sequencing primer from the 5′ UTR of *Tccn* was used to obtain 1 kb of upstream genomic sequence from one of the positive clones. This sequence was analyzed for the presence of promoter elements (Figure 5). The Drosophila *cinnabar* (*cn*) gene is known to possess consensus CAAT and TATA boxes (WARREN *et al.* 1996), the latter being located 134 bp upstream of the translation start site. While consensus CAAT and TATA boxes were found 1 kb upstream of the 5′ end of the *Tccn* cDNA (Figure 5), analysis of the downstream sequence failed to reveal similarity to the amino-terminal extension observed in the Drosophila protein. No promoter motifs were detected in the intervening sequence. However, nine consensus Inrs (CHERBAS and CHERBAS 1993) were identified between −170 and +1 (+1 is the 5′-most nucleotide of pC5′cn; Figure 5). These observations and the similarity in length, N-terminal to a region of sequence alignment, between the deduced amino acid sequences of *Tccn* and human and silkworm KM proteins suggest that the *Tccn* cDNA includes the complete coding region.

Correlation between larval eyespot pigmentation and adult eye color: Tribolium larvae lack compound eyes. However, pigmented eyespots are visible in wild-type larvae and late-stage embryos. Mutants such as *vʷ*, *pearl*, and *platinum*, which exhibit white-eyed adult phenotypes, also lack larval eyespot pigmentation (see Table 1). The same is true for mutants with very light adult eye-color phenotypes, such as *peach*, *pink*, and *ivory*. Conversely, mutants such as *hazel*, *ruby*, and *chestnut* that have a less severe effect on adult eye color also retain detectable pigmentation in the larval eyespots. Thus, larval eyespot pigmentation is well correlated with adult eye color, and lack of larval eyespot pigmentation predicts a drastic reduction or complete loss of adult eye color. In the experiments described below, we use larval eyespot pigmentation to assess the effects of RNA interference (RNAi) and identify candidate mutants that might correspond to cloned eye-color genes.

RNA interference: Either *Tcv* or *Tccn* dsRNA was injected into wild-type embryos to determine their loss-of-function phenotypes. After *Tcv* injection, 11 of 22 late-stage embryos and newly hatched larvae lacked eyespot pigmentation (Figure 7, A and B). Injection of *Tccn* dsRNA resulted in complete loss of eyespot pigmentation in 6 of 19 newly hatched larvae (Figure 7C) and dramatically reduced pigmentation in 11 others. In both cases, the effect was transient, since wild-type eye color developed during pupation. Eyespot pigmentation was never affected by injection of dsRNA from genes not involved in eye-color pigmentation (>500 hatchlings examined) or mock injections with buffer alone (>1000 hatchlings examined). These results demonstrate that the *Tcv* and *Tccn* orthologs affect eye pigmentation in Tribolium and predict a null mutant phenotype with little or no adult eye color.

-885   -841   -822

```
CTCAAAAGTGTCTTCGTAAGCAAACTAATCACCTACTTCATCTACAATATTTTCAGTTACCCATTATAAAA

ATTGATTTTTCTTCACAGTTACTGTTCTTCTTTTTCAACTTTTTTAATGTATTCGGTTAATAATATTCGGA

TGTTTTATTTATTTTCTTTTTTGTCAATTTGATAAAATTGATTTTGATACTAGTCAGTAGATGATCGGACG

GTCTATATTTATTTGGTGACCAAAGCTTTTTTTCTTCATTCACTCGAAGATAAAGAAGTAGTCAGTGCGGA

GAGCTCAGTAGAAGAGAACTGAAGAAGACGTTAGCTTTAGCTCTACAAGCCAGAATTTTTTTCTTATACCT

TATATAGTATCTTCTTTGCCAAAAGCAGAAAAATGAAATAGTGAAAAATTTGGAATACTACTTCAGGAATC

TGGAAAAGCTGGACAAAAGACAAAAAGTGTTTGAAATTTGTTGATTCCTCGTTAGTGAGGAAGAACAAAT

AGAAAGTGATGATGAAGAAGACGCTTTTTGTACATTCTGCAAAATCCTCTTTTTTTGAAAGTGATTTTGAA

AAGTGAATACGTGTACTACGAGTAACTAAAAAATGGGCTACTGATGTGACACCAGTCAGCATCGTCACCAA

CACTGTTTCTGCTTTTGATTATTTGGATGTGTTAAATAAATAAGAGATAACAAACTTGTTTATGATTAGGA

ACATTTCAGAAATCACAGTAGATTAATGAGTGGAATTTTTCTATTTATTTTGCCATCAATTGCACAAGAAA
```
  -100   -80   -60   -40
```
CTTATCATAGAATTACTCGGATTAGCATTGAGAAAGAAAAAGGCGCAAGCCCCGAAATGTAACACAACACT
```
     -20   +1
```
AACACGATTATTCTACATATCAAGTGGCTGAAACGAAGCCTTTAAACTAGAACCAGTCCTGTGTTGGTGGT

TTTTGTGTGAAAATGGGTAGTAATAACGTCAATTCGGTTATTGTAGTGGGCGGTGGTTTGgtatggcccta

aatgcaccaaaacaaaaataaaattgattttttaagGTTGGTTCTTTGTGTGCTATTTTTATGGCAAAGAG

GGGCTACCATGTTACACTTTTTGAGTATCGGGAAGgtaaattaattttttaatgagattagggccgaactt

tttgatttagATATTAGGACGGCAAAGTTTGCTCGTGGCCGTTCGATAAATATGGCTTTGTCGAACCGTGG

CAGAAAAGCGCTACGTGCTGTTGGAATAGAAAAAATAATTCTTGAATCGGCGATTCCTATGAAAGGGCGGC

TTTTACACGACTTGAAGGGGCGAACCACTAGTGTACCATACGATGCTTTAACGGGACAAgtacctggttaa

tttgcccataaaacttgtgtttattgtaaaaaattctagTGTATTTATTCGATCAGCAGAGATTACTTAAA

TAACGTGCTGTTAACCGAATTAGAGAAGTACCCAAATGTGAAAATTTATTTCAATCATAAATTAATGAGTG

TGAGCTTTGAGGATGAGAGAATTTCGGTCATGAAgtaggtaaaaaatgtccaaaacctaaaatcaataatt

tttatagTTTGATAACTGAAGAAATTACGACCCACCAAGCCGATTTAATAATAGAGGCCGATGGGGCCTAC

AGTACCCTTCGGCGTTACATGCAGCTGACCCCTCTTTTTGAGTACAGCCAAACCTACATCCAACACGGGTA

CCTCGAGTTGGTCATCCCTCCCGAAAACGGGCCCAAAATGACCCCCAATCATCTACACATCTGGCCCCGGG

GCCAATTCATGATGATTGCGTTACCCAACAAGGACAATTCCTGGACTGTCACCCTGTTTATGCCTTTTGGT

AAATTCGAAAGTTTGAGAAATGCTGCAGAATTGAAAGATTTTTACTACAAGACTTTCCCCGACGCTGTCCC

CCTCATCGGTGAGGATCTCCTCGTGAACGATTTTTTTAAAGTGAAGCCATCGGCTCTGGTCTCGGTCAAGT

GTAAACCGTACCATGTGGGGAGCAAATTTCTGCTGATTGGGGACGCGGCACATGCCATGGTACCCTTTTAT

GGGCAGGGGATGAATGCAGGGTTTGAGGATTGTTTCCTTTTGGACGGCATTCTAGAAAGGAGGAGTAACGA

CATCGCGGGGTCTATTGAGGAGTTCAGTAGGGAACGGGTTGAGGATGCTTATGCTATTTGCGAGTTGGCGA

TGTATAATTACGTGGAAATGAGGGATCTTGTGACTAGGCCTTCGTACCGTTTGAGAAAATTTTTCGATGAA

TTGCTTTTTAAGTGTATGAAGGAGAAGTGGATTCCGTTGTGCAATTCGGTGACTTTTTCGAATTTCGGGTA

CAAGCAGTGTGTGGAAAACAGGAAGTGGCAAAACAAGgtttgtggacacgtaattgggatcaatatgaaac

aagtttttagGTTATTCAAAAATTTCTCTGGACGGGTGGCTTGGTAACTAGCGCTTTGTTTGCATATGGATT

ACGGATTTGGTTGTATTAAAATATTTGCGTTTTTTATCAGTTTAATTTATTGTAAGACTTTTCTGAATAAA
```

FIGURE 5.—Nucleotide sequence of the wild-type *Tc cinnabar* locus. +1 indicates the start of the longest *Tccn* transcript detected. In the putative *Tccn* promoter (−885 to +1) consensus TATA and CAAT boxes are in boldface type, while possible initiator sequences are underlined. Start and stop codons are boxed. Introns are shown in lowercase italics. The polyadenylation signal is double underlined. GenBank accession nos. are AYO52393 (pGcn2) and AF422805 (putative *Tccn* promoter).

```
Tc  ------------------------------------------------------------------
Dm  MFVATPLHLVGFPQQHQTTIDTIYCPSDIISWLNISPASCLASISIPSSFRCQRASRTVMSPGIVSQ
Bm  ------------------------------------------------------------------
Hs  ------------------------------------------------------------------


Tc  -------------MGSNNVNSVIVVGGGLVGSLCAIFMAKRGYHVTLFEYREDIRTAKFARGRSIN
Dm  EVNGRQEPTEAARDERHGRRRRVAVIGAGLVGSLAALNFARMGNHVDLYEYREDIRQALVVQGRSIN
Bm  ---------MANLENGTFKKLDVVVVGGGLVGSLEALFLAKRGHRVRLYEYREDIRNTPQARGRSIN
Hs  -----------MDSSVIQRKKVAVIGGGLVGSLQACFLAKRNFQIDVYEAREDTRVATFTRGRSIN


Tc  MALSNRGRKALRAVGIEKIILES-AIPMKGRLLHDLKGRTTSVPYDALTGQCIYSISRNYLNNVLLT
Dm  LALSQRGRKALAAVGLEQEVLAT-AIPMRGRMLHDVRGNSSVVLYDPINNQCLYSVGRRQLNEVLLN
Bm  LALSIRGRTALREVGLEDHMINNHGIPMKGRNIHRIDGSTYIIPYDSRTKQCIYSVGRNYLNGLLLQ
Hs  LALSHRGRQALKAVGLEDQIVSQ-GIPMRARMIHSLSGKKSAIPYGTKS-QYILSVSRENLNKDLLT


Tc  ELEKYPNVKIYFNHKLMSVSFEDERISVMNLITEEITTHQADLIIGADGAYSTLRRYMQLTPLFEYS
Dm  ACDKLPNIRCHFEHKLTSANLREGSMEFRN-PAKEAVAAHADLIVGCDGAFSSVRQHLVRLPGFNYS
Bm  ESEKYENVERFFNHKLIASNLRKGFLSFQKTDTKEIVEVNADLIIGADGAFSAVRKEIMKQPLFDYN
Hs  AAEKYPNVKMHFNHRLLKCNPEEGMITVLG-SDKVPKDVTCDLIVGCDGAYSTVRSHLMKKPRFDYS


Tc  QTYIQHGYLELVIPPE-NG-PKMTPNHLHIWPRGQFMMIALPNKDNSWTVTLFMPFAKFESLRNAAE
Dm  QEYIETGYLELCIPSKS-GDFQMPANYLHIWPRNTFMMIALPNQDKSFTVTLSMPFEIFAGIQNQND
Bm  QKYIEHGYLELCIPPDSNGGFQMPPNYLHIWPRGEFMMIALPNQDCSWTVTLFMPFTHFKSLDNEDK
Hs  QQYIPHGYMELTIPPK-NGDYAMEPNYLHIWPRNTFMMIALPNMNKSFTCTLFMPFEEFEKLLTSND


Tc  LKDFYYKTFPDAVPLIGEDLLVNDFFKVKPSALVSVKCKPYHVGSKFLLIGDAAHAMVPFYGQGMNA
Dm  LLEFFKLNFRDALPLIGEQQLIKDFFKTRPQFLVSIKCRPYHYADKALILGDAAHAMVPFYGQGMNA
Bm  LLKFFEKYFPDSIPLIGKQKLIADYFAGSASPLIAIKCRPYNVEDKALIIGDAAHAVVPFYGQGMNA
Hs  VVDFFQKYFPDAIPLIGEKLLVQDFFLLPAQPMISVKCSSFHFKSHCVLLGDAAHAIVPFFGQGMNA


Tc  GFEDCFLLDGILERRSNDIAGSIEEFSRERVEDAYAICELAMYNYVEMRDLVTRPSYRLRKFFDELL
Dm  GMEDVTLLTDILAKQLP-LDETLALFTESRWQDAFAICDLAMYNYVEMRDLTKRWTFRLRKWLDTLL
Bm  GFEDCTILNQLFQKHHDDLAKILKEFSDTRWEDTFAISDLAMYNYIEMRDLVTRPSYLLRKAIDDVI
Hs  GFEDCLVFDELMDKFSNDLSLCLPVFSRLRIPDDHAISDLSMYNYIEMRAHVNSSWFIFQKNMERFL


Tc  FKCMKEKWIPLYNSVTFSNFGYKQCVENRKWQNKVIQKFLWTGGLVTSALFAYGLRIWLY----445
Dm  FRLFP-GWIPLYNSVSFSSMPYRQCIANRKWQDQLLKRIFGATFLAAIVTGGAIYAQRFL----524
Bm  FWLMPKVWIPLYNSVTFSTIPYTQCIKNRQWQDKVLNRSLLFLGTVTSAVGAFYVYKKFVGLS-365
Hs  HAIMPSTFIPLYTMVTFSRIRYHEAVQRWHWQKKVINKGLFFLGSLIAISSTYLLIHYMSPRSFLCL


Hs  RRPWNWIAHFRNTTCFPAKAVDSLEQISNLISR-----------------------------486
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FIGURE 6.—Multiple alignment of kynurenine 3-monooxygenase sequences from *Tribolium castaneum* (*Tc*; GenBank accession no. AYO52391), *Drosophila melanogaster* (*Dm*; GenBank accession no. U56245), *Bombyx mori* (*Bm*; GenBank accession no. BAB62419), and *Homo sapiens* (*Hs*; GenBank accession no. AF056032). Residues that are identical in all four species are in boldface type, and regions used for designing degenerate primers are underlined.

***Tcv* is tightly linked to *white*:** *Tcv*-related dimorphisms between eye-color mutant and wild-type (T-1) strains were identified by agarose gel or single-strand conformation polymorphism (SSCP) analysis of PCR products. These dimorphisms were used to assess linkage between *Tcv* and candidate eye-color mutations. Several eye-color mutations assorted independently of the *Tcv*-related dimorphism and are therefore eliminated as candidate alleles of the *Tcv* gene (data not shown). However, the *h* and v^w mutations showed linkage to *Tcv*. Recombination occurred between *Tcv* and *h* in 3.4% (3/87) of the backcross progeny. No recombination (0/72) occurred between *Tcv* and the v^w mutation. Thus, recombinational mapping identifies v^w as the best candidate *Tcv* mutant.

Most of the *Tcv* locus is deleted in v^w beetles: Genomic DNA from the v^w strain was analyzed by Southern hybridization for polymorphisms associated with the *Tcv* locus. A probe consisting of the 1.8-kb genomic fragment pGv2, which contains the entire *Tcv* coding region, identified a single, 8-kb *Eco*RI fragment in the wild-type GA-1 strain and the *p* and *h* mutant strains. However, the *Eco*RI fragment identified in v^w DNA was >12 kb (Figure 8A). A second probe, complementary to the 3′ end of the *Tcv* coding region, produced results identical to those seen with the full-length fragment (data not shown). However, a 900-bp fragment containing only the *Tcv* promoter and 5′ coding region (exons 1–2 and part of 3), hybridized to wild-type, *h*, and *p* DNAs, but
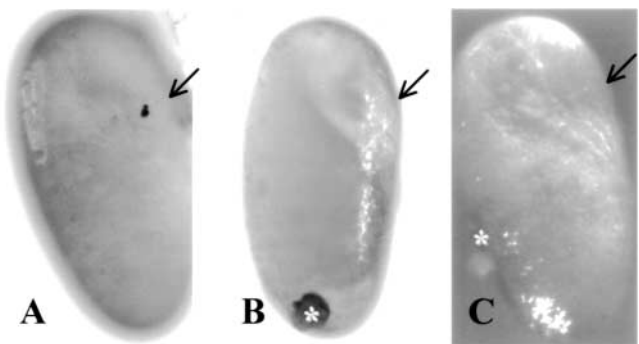

FIGURE 7.—Effect of *Tcv* and *Tccn* RNA interference on eyespot pigmentation in *Tribolium castaneum*. (A–C) Lateral views of late-stage GA-1 embryos illustrate the effect of *Tcv* or *Tccn* dsRNA. (A) Wild-type eyespot pigmentation observed in late-stage GA-1 embryo. (B) A GA-1 embryo of the same age, but previousy injected with *Tcv* dsRNA. (C) A GA-1 embryo of the same age, but previously injected with *Tccn* dsRNA. Asterisks indicate the sites of injection (B, posterior; C, midline). Arrows indicate the locations of larval eyespots on the dorsal, lateral side of the head.
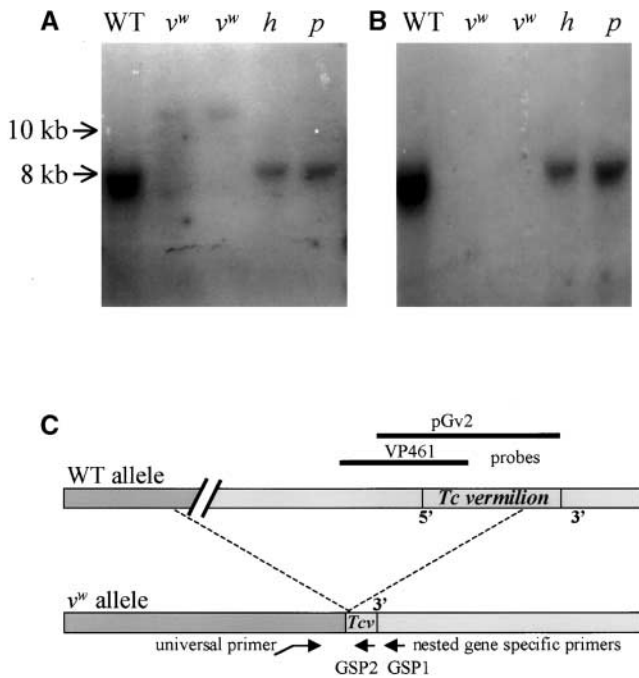
FIGURE 8.—Southern hybridization analysis of the *Tc vermilion* (*Tcv*) locus in strains of *Tribolium castaneum*. (A) Autoradiogram of blot after hybridization to the full-length *Tcv* probe pGv2. (B) Autoradiogram of the same blot after hybridization to a *Tcv* probe (VP461) containing the *Tcv* promoter and 5′ coding region only (exons 1–2 and part of 3). Wild-type DNA (WT), *vermilion^{white}* DNA (*v^w*), *hazel* DNA (*h*), and *pearl* (*p*) DNA are shown. (C) Amplification of the *vermilion^{white}* deletion breakpoint by universal PCR. GSP1 and GSP2 indicate the nested *Tcv*-specific primers complementary to a region near the 5′ end of exon 6 and with their 3′ ends facing upstream toward the deletion breakpoint. While the highly degenerate universal primer [uni-7, GGGTTTTCCAGTCACGAC(N)$_8$GGA TCC] can anneal to numerous sites within the genome, only those that anneal within ~1 kb upstream of the GSP primers will lead to amplification of the *v^w* breakpoint. Slash marks indicate that the exact size of the deletion is unknown. The positions of the probes used for Southern analysis are indicated above the WT allele.

failed to hybridize to *v^w* DNA (Figure 8B). These results suggest that *v^w* beetles are homozygous for a deletion that removes the 5′ end of *Tcv*.

To confirm this, the putative *Tcv* deletion breakpoint was amplified from *v^w* genomic DNA via universal PCR (Figure 8C). Sequence analysis of the PCR product (pGv^w; GenBank accession no. AF419848) confirmed the presence of a rearrangement breakpoint in exon 6 (see Figure 2). The sequence immediately flanking the upstream side of the breakpoint is not derived from the *Tcv* locus. As expected, PCR primer pairs targeted to regions of the *Tcv* transcription unit upstream of the *v^w* breakpoint amplify the expected fragment sizes from several wild-type strains but failed to produce a product from the *v^w* strain (data not shown). Thus, ~80% of the *Tcv* coding region as well as the putative promoter,

transcription start site, and translation start site are deleted in *v^w* beetles.

Tccn is unlinked to known eye-color mutants: Linkage between *Tccn* and candidate eye-color mutations was assessed by using *Tccn*-related dimorphisms between eye-color mutant and wild-type (T-1) strains identified by PCR or SSCP analysis. All eye-color mutations tested assorted independently of the dimorphisms (data not shown). Moreover, further mapping demonstrated that *Tccn* is located on linkage group 2 (R. W. BEEMAN, unpublished results) for which there is no known eye-color mutant (see Table 1).

DISCUSSION

Eye pigmentation in Tribolium: Orthologs of the Drosophila eye-color genes *vermilion* and *cinnabar* were initially identified by degenerate PCR and further characterized by analysis of genomic and cDNA clones. In addition, a molecular lesion at the *Tcv* locus was identified in the white-eyed mutant *v^w*. *Tcv* encodes Tryptophan oxygenase, the first enzyme in the ommochrome pathway.

Both pteridine and ommochrome pigments span a wide color spectrum in different insect species (LINZEN 1974). In *Drosophila melanogaster*, both the brown ommochrome pigments and the reddish pteridine pigments are required to confer the red-brown color of the wild-type eye of this species. The loss of *vermilion* and concomitant knockout of the ommochrome pathway result in red-eyed flies. In contrast, the loss of ommochrome pigments via *Tcv* knockout in *T. castaneum* results in a complete loss of eye pigmentation, suggesting the presence of a single pigmentation pathway in this species. Several lines of evidence suggest that red ommochrome pigments are the principal contributors to the black eye color of wild-type *T. castaneum*. First, the available mutants display various shades of red eye color (see Table 1). Second, rescue of *v^w* beetles by transient expression of *Tcv* results in pink to red adult eye color (M. D. LORENZEN, unpublished results). Finally, in independent transgenic lines, *Tcv* transgene expression in white-eyed *v^w* recipients results in pink, red, or black adult eye color (M. D. LORENZEN, unpublished results).

Taken together, the phenotypes produced by *Tcv* RNAi, *Tcv* rescue, and the *v^w* and other eye-color mutations suggest that the Tribolium larval eyespot and adult eye are pigmented only by red ommochromes, and that pteridine pigments do not contribute to eye color. This conclusion is not unprecedented, since the compound eyes of at least one other insect species (*A. gambiae*) are colored by ommochrome pigments only (BEARD *et al.* 1995).

Eye-color genes as transformation markers: Despite much effort, reliable and versatile methods for germline transformation in nondrosophilid insects have remained elusive. One obstacle has been the lack of efficient pheno-

typic markers for transformant identification. Most reports of nondrosophilid insect transformation have employed either *white* (ABC transporter) or *GFP* markers (Atkinson *et al.* 2001). EGFP is functional in all species tested and obviates the need for mutant recipient strains, but requires specialized detection systems. While dipteran *white* (*w*), *cinnabar* (*cn*), and *vermilion* (*v*) genes have not been demonstrated to function outside their taxonomic order of origin, they do show some ability to function in different dipteran families (White *et al.* 1996; Cornel *et al.* 1997; Coates *et al.* 1998; Jasinskiene *et al.* 1998; Atkinson *et al.* 2001). This observation raises the hope that *T. castaneum* eye-color genes might function as broad-spectrum transformation markers in the order Coleoptera. With the cloning of the Tribolium ortholog of *vermilion* and the identification of a molecular lesion that eliminates most of the *Tcv* locus, we now have the components necessary to construct a transformation marker system on the basis of eye-color rescue. A separate report will summarize our recent efforts to develop *piggyBac* and *Hermes*-based transformation systems that utilize *Tcv* for transformant identification in Tribolium.

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